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Cross linking of deoxyribonucleic acid in furazolidone treated *Vibrio cholerae* cell

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The broad spectrum antimicrobial compound furazolidone or *N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone was shown to inhibit DNA synthesis in *V. cholerae* cells while stimulating RNA and protein synthesis at the same time and causing filamentation of the cells [1, 2]. Synthesis of the cholera phage ϕ 149 DNA was at least ten times more sensitive to this inhibitory action of furazolidone [3, 4]. The drug was found to bind with DNA *in vitro* by intercalation between the base pairs, thereby causing hindrance to DNA strand separation and inhibition to the digestion of DNA by DNase [5]. Whether this intercalative process of binding of furazolidone with DNA is responsible for the inhibition of DNA synthesis *in vivo* or whether furazolidone undergoes some metabolic changes or activation *in vivo* and the resulting product is responsible for the inhibition of DNA biosynthesis either by binding directly with DNA or by some other process has so far been a subject of speculation. This communication reports in this respect that inter strand cross linking in DNA takes place within the furazolidone treated *V. cholerae* cells, which might explain the actual mechanism of inhibition of DNA biosynthesis by this drug.

V. cholerae cells, OGAWA 154, were grown in the logarithmic phase in absence or presence of 0.5 μg furazolidone/ml. At different times, bacteria were harvested, washed in physiological saline and DNA was extracted following in general the method of Marmur [6]. DNA thus isolated from drug treated or untreated bacteria was essentially free of RNA and protein [5]. Melting temperature (T_m) of DNA isolated from furazolidone treated or untreated cells was determined by a Hilger Watt spectrophotometer providing a thermostat controlled cell holder. Cross linking of DNA was estimated from the fraction of DNA which has been rendered reversibly renaturable following thermal denaturation in accordance with the general procedure of Geiduschek [7], as described by Rutman *et al.* [8], and by using the formula,

$$\% \text{ Reversibly renaturable DNA} = \left[1 - \frac{(A_d - A_n)_{alk}}{(A_d - A_n)_{cont}}\right] \times 100$$

where A_d, A_n are the absorbances at 25 C of denatured and native DNA in the alkylated and control samples. For study of recovery, cells treated with the drug for 45 min in the logarithmic phase of growth were centrifuged, washed and resuspended in phosphate buffered saline (0.15 M), pH 8.0. The organisms were then serially diluted in the same buffer and stored at 37° in the dark for different periods up to 24 hr. After the desired period of holding, platings were done on nutrient agar surface and the resulting colonies were counted after overnight incubation at 37°.

A very high percentage of reversibly renaturable DNA was detected in DNA isolated from furazolidone treated cells, as compared to the DNA from untreated cells, indicating that inter strand cross links resulted from drug treatment. The per cent reversibility of renaturation depended significantly on the period for which the log phase cells were treated with the drug. For 30 min treatment, the reversibility varied between 64 and 67 per cent in several different experiments, while it ranged between 85 and 92 per cent when the drug treatment was continued for 45 min. This may be compared with the 7–14 per cent reversibility obtained for DNA isolated from untreated bacteria and also for native DNA treated *in vitro* with furazolidone. The *in vitro* and *in vivo* actions of furazolidone are thus different. The isolation of such denaturation resistant DNA is considered as diagnostic of cross linking since the presence of a covalent link permits the two strands to "snap back" into the correct complementary structure after heat denaturation [9]. The *in vivo* action of furazolidone has considerable similarity with that of mitomycin-C [10, 11]. Both the agents induce inter strand cross linking in DNA *in vivo*, inhibit DNA synthesis and cause filamentation of the cells at the appropriate dose level by inhibiting cell division. Lawley and Brookes [12] reported that cross links involving alkylation of the N-7 position of guanine were thermally quite unstable because of the simultaneous labilization of the purine deoxyribose bond. Mitomycin induced cross links were, on the other hand, thermally stable and conferred resistance on the DNA double helix to thermal denaturation [11]. Furazolidone-induced cross links also enhanced the thermal stability of DNA and the melting temperature (T_m) of DNA was raised from 69° (for native DNA under identical condition) to 77.5° (Fig. 1). The significant point of difference noted so far is that unlike mitomycin-C, furazolidone binds with DNA *in vitro* by an intercalative process. It is of interest to note that the antibiotic carzinophillin also inactivates by the formation of cross links as shown by the denaturation resistance of treated *B. subtilis* transforming DNA [13].

Effects of furazolidone treatment on the viability and macromolecular synthesis in actively growing cells of *V. cholerae* were reported earlier in detail [1, 2]. 45 min treatment by 0.5 μg furazolidone/ml resulted in a 10–15-fold drop in viability of the cells. However, the cells could

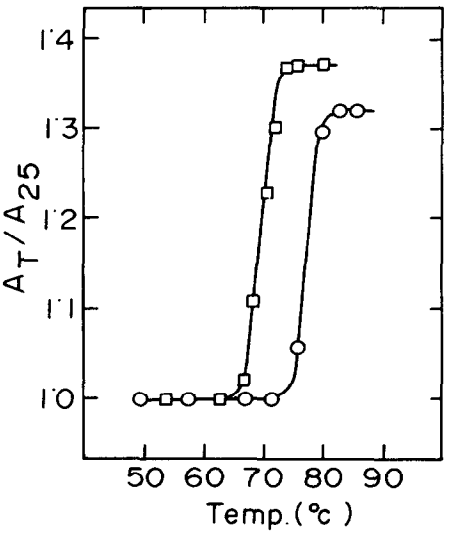

Fig. 1. Thermal transition profiles in 5 mM Tris–HCl, pH 7.4 of DNA isolated from log phase *V. cholerae* cells treated with 0.5 μg furazolidone/ml for 30 min (○); Transition profile for DNA (control) from untreated cells (□).

recover significantly when incubated in buffer at 37° in the dark and there is evidence indicating removal of most of the cross links in DNA by such treatment. Untreated cells proved wholly stable in this buffer at 37° showing no significant fluctuation in viability count up to at least 24 hr. Treated cells showed no significant rise in viability count up to the first 2 or 3 hr but thereafter the count started rising. After 18, 21 and 24 hr holding in the dark at 37°, the viability counts increased 8-, 9- and 13-fold respectively (relative to the ohr. holding of the washed cells). This is comparable to the recovery in mustard gas treated *Escherichia coli* strain B [14]. A major discovery by Lawley and Brookes [15, 16] and by Kohne, Steibigel and Spears [17] was the finding that cross links appear to be removed from the DNA of *E. coli* cells and that this process is controlled by the uvrB gene. This removal could be demonstrated by several techniques, one of which was the loss of the reversibly bihelical property of DNA [18]. DNA isolated from furazolidone treated cells held in buffer for 18 hr at 37° in dark exhibited only 17–25 per cent of reversibility in renaturation indicating removal of most of the cross links.

Cross linkages between the complementary strands of DNA *in vitro* or in cells were detected after treatment with several agents including X-rays [19], nitrous acid [20], nitrogen or sulfur mustards [21], mitomycin-C [10, 11] etc. and several mechanisms for the cellular repair of cross linked DNA have been suggested [18]. Excision and genetic recombination have apparently been evident in contributing to recovery from damages induced by cross linking agents in bacterial cells [18]. But the exact mechanism of repair of the cross linked DNA in furazolidone treated cells has yet to be investigated. Also the chemical nature of the cross linking agent derived from the biotransformation of furazolidone remains to be known.

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